PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	Group Art Unit: 1644
KUNDIG and McCORMACK	)	
	)	Examiner: Phyong N. HUYNH
Serial No.: 09/804,464	)	
	)	Docket No: 005184.00002
Filed: March 13, 2001	)	

For: **MODULATION OF ALLERGIC RESPONSE**

DECLARATION OF DR. THOMAS KÜNDIG UNDER 37 C.F.R. § 1.132

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Thomas Kündig, declare as follows:

1. I am named as an inventor of the subject matter claimed in the application referenced above.

2. I hold an M.D. degree from the University of Zürich and have worked in the fields of immunology and allergy for 14 years. My curriculum vitae is attached as Exhibit 1.

3. We conducted a clinical study that included a total of 153 human hay fever sufferers. Each of these patients was allergic to grass pollen, as assessed by skin prick testing to grass pollen extract. Patients were randomized into two study groups. Eighty-six patients in the conventional treatment group received conventional grass pollen immunotherapy. These patients were subcutaneously injected with 16 incremental doses of grass pollen extract over 20 weeks, followed by twice-weekly subcutaneous injections of a maintenance dose corresponding to 100

μg alum-absorbed grass pollen extract (100,000 so called "SQ-E units"). Sixty-seven patients in the intralymphatic group received three injections of 1 μg of the same alum-absorbed grass pollen extract into a superficial subcutaneous lymph node of the groin spaced four weeks apart. The efficacy of both the conventional and the intralymphatic treatments were assessed four months after the initial treatment. The protocol is shown in Exhibit 2.

4. To assess efficacy, patients in both treatment groups were evaluated for their sensitivity to the allergen by nasal provocation with an extract of the same pollen extract. Fifty μl of pollen extract were administered under rhinoscopic control onto the mucosa of the lower concha. A first dose of 100 SQ-E (approximately 0.1 μg of grass pollen extract) was followed after 30 minutes by 1,000 SQ-E (approximately 1 μg), then by 10,000 SQ-E (approximately 10 μg) and finally by 100,000 SQ-E (approximately 100 μg) after 60 and 90 minutes, respectively. The following symptoms and scores were recorded: ocular or nasal itching (0 = none; 1 = mild, *i.e.*, slight sensation; 2 = moderate, *i.e.*, definite sensation; and 3 = severe, *i.e.*, need to rub nose or eyes); runny nose (0= none; 1 = mild, *i.e.*, slight sensation; 2= moderate, *i.e.*, definite sensation; and 3 = severe, *i.e.*, need to blow or wipe nose); nasal congestion (0= none; 1= mild, *i.e.*, slight sensation; 2 = moderate, *i.e.*, definite sensation; and 3= severe, difficult to breathe through one or both nares); and sneezing (0= none; 1= mild, *i.e.*, 1-2 sneezes; 2 = moderate, *i.e.*, 3-4 sneezes; and 3= severe, *i.e.*, 5 or more sneezes). The results are shown in Exhibit 3.

5. The upper left panel of Exhibit 3 depicts the symptom scores observed after nasal provocation with 100 SQ-E units (approx. 0.1 μg of pollen extract). At this low pollen concentration the intranodal group shows fewer symptoms after treatment, but due to the generally low symptom scores at this low pollen concentration, the latter improvement of the intranodal group shows no statistical significance (p= 0.45). The upper right panel of Exhibit 3

shows the symptom scores after nasal provocation with 1,000 SQ-E units (approximately 1 μg of pollen extract). Again the intranodal group of patients shows a lower symptom score after treatment, but again the overall symptom scores are low, so that the improvement is not statistically significant (p=0.07).

6. The lower left panel of Exhibit 3 shows the symptom scores after nasal provocation with 10,000 SQ-E units (approximately 10 μg of pollen extract). Because of the higher pollen concentration, the overall symptom scores are higher and the improvement of the intranodal group becomes statistically significant (average score before treatment 4.47, compared with 1.9 after treatment). The lower right panel of Exhibit 3 shows the symptom scores after nasal provocation with 100,000 SQ-E grass pollen extract (approximately 100 μg pollen extract). Before treatment the intranodal group showed an average symptom score of 9.63; after treatment, the symptom score is reduced to 5.05. This change is statistically highly significant (p=0.002).

7. Thus, intralymphatic administration of allergen administration is more efficient than subcutaneous administration of the same allergen. No more than three intranodal injections of an allergen are sufficient to desensitize human patients to the allergen, as assessed by sensitivity to the allergen. This permits a reduced number of injections and therefore substantially shortens the duration of treatment.

8. Patients that were desensitized by only 3 intranodal injections were assessed by nasal provocations tests 4 months after the beginning of treatment. As shown in Exhibit 4, this patient group tolerated approximately 10 times higher pollen concentrations after treatment. In contrast, subcutaneously desensitized patients showed no significant improvement of their nasal provocation thresholds. In order to achieve a 10-fold increase in the nasal provocation threshold

conventional subcutaneous immunotherapy usually has to be continued for 3 years, as demonstrated by Wihl *et al.* for tree pollen (*Allergy 43*, 363-69, 1988; Exhibit 6). Similarly, Pegelow *et al.* (*Allergy 39*, 275-90, 1984; Exhibit 7) showed that after 3 years of immunotherapy with grass pollen the average increase in the nasal provocation threshold is not more than a factor of 10.

9. It is unexpected that treatment with merely three injections of 1 μg more efficiently reduces the symptom score than the extensive conventional subcutaneous regimen with 20 incremental injections up to 100 μg of the same pollen extract. Based on these results, I would expect that the efficiency of desensitization with other allergens such as venoms, animal dander, and dust mite would be similarly enhanced by intranodal administration.

10. Pollen-specific IgG also was measured before treatment and four months after the beginning of treatment. No significant changes were observed. Amongst all IgG subclasses, subclass IgG4, if at all, is the one subclass where increases have been reported by other groups. The graph shown in Exhibit 5 depicts specific IgG4 against the major allergenic grass in Europe (*i.e.*, *Phleum pratensae*) in patients treated by intralymphatic allergen injections. IgG4 titers were measured by the Pharmacia CAP™ method, which permits a quantitative assessment. There was no significant titer change in *Phleum pratensae*-specific IgG4 before and after treatment. Thus, the mechanism of intralymphatic desensitization does not depend on induction of allergen-specific IgG4 antibodies.

11. A total of 20 systemic allergic adverse events were observed during a one hour post-injection wait for the conventional group. Two of these events (asthma attacks) were classified as severe (grade 3 according to H.L. Müller, *New Engl. J. Med. 261*, 374-77, 1959; J. *Asthma Res. 3*, 331-33, 1966). Eighteen of these events were classified as mild or moderate

or angioedema. In contrast, only six systemic allergic events were observed during a two hour post-injection wait for the intralymphatic group. These patients suffered from flush, urticaria or angioedema (grades 1 and 2 according to H.L. Müller). This demonstrates that the frequency and severity of adverse events are reduced with intralymphatic treatment compared to conventional treatment.

12. At the one year follow up visit, 40 of the original 86 patients in the conventional treatment group had dropped out of the study. In contrast, only three of the original 67 patients in the intralymphatic treatment group had dropped out. Thus, patients receiving intralymphatic treatment show better compliance and are more likely to finish their treatment.

13. I declare that all statements made herein of my own knowledge are true and that I believe all statements made on information and belief are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

_____ _____

Date Thomas Kündig, M.D.

EXHIBIT 1

Curriculum vitae of Thomas Kündig

Name:	Kündig
First Name, Middle Name:	Thomas, Martin
Titels:	PD Dr.med.
Birth data:	22.1.1963
Nationality:	Schwitzerland and USA
Working place:	Dermatologische Klinik Gloriastr. 31 8091 Zürich Tel: 0041-1-255-3973 FAX: 0041-1-255-4418

Achievements:

1981 Graduation
Mathemathical-Scientific type (Typus C),
Kantonsschule Zürcher Oberland

1987 MD degree
University of Zürich

1988 Medical Thesis
in respiratory physiology, supervisor Prof. E.A. Koller
University of Zürich

1988 Educational Commission for Foreign Medical Graduates
Basic Sciences, Clinical Sciences and English
Bern

1991 Post-graduate Course in Experimental Medicine
especially Immunology, supervisor Prof. R.M. Zinkernagel
Universität Zürich

1998 Venia legendi in Experimental Immunology and Experimental Dermatology

Working Places:

Kreisspital Wetzikon Dr. R. Stahel	Resident	1988-89	Surgery
Inst. for Experimental Immunology Universität Zürich Prof. R.M. Zinkernagel	Post-doc	1989-92	Immunology
Dept. of Medical Biophysics University of Toronto Prof. T.W.Mak	Post-doc	1992-95	Immunology
Dermatologische Klinik Universität Zürich Prof. G. Burg	Oberassistent Oberarzt	1995-99 1999-	Dermatology

Awards and Honors:

Award for best Marks at Graduation Exams 1987
Fellowship for Post-graduate Course in Experimental Medicine 1989-91.
Fellowship as an advanced researcher by Swiss National Science Foundation 1992-94.
Venia legendi in Experimental Immunology and Experimental Dermatology 1998
Georg-Friedrich Götz Award by the University of Zürich 1999 for outstanding medical research on the development of a new method to enhance the immunogenicity of vaccines
Affiliated Researcher of the Ludwig Institute for Cancer Research

Publications (Original):

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Kozieradzki-I; **Kundig-T**; Kishihara-K; Ong-CJ; Chiu-D; Wallace-VA; Kawai-K; Timms-E; Ionescu-J; Ohashi-P; Marth-JD; Mak-TW; Penninger-JM
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Mittrucker-HW; Matsuyama-T; Grossman-A; **Kundig-TM**; Potter-J; Shahinian-A; Wakeham-A; Patterson-B; Ohashi-PS; Mak-TW
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Schmits-R; Filmus-J; Gerwin-N; Senaldi-G; Kiefer-F; **Kundig-T**; Wakeham-A; Shahinian-A; Catzavelos-C; Rak-J; Furlonger-C; Zakarian-A; Simard-JJ; Ohashi-PS; Paige-CJ; Gutierrez-Ramos-JC; Mak-TW
CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity.
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Am-J-Dermatopathol. 1997 Aug; 19(4): 391-5

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Lupus profundus/panniculitis
Dermatology. 1997; 195(1): 99-101

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[Dapsone in granulomatous rosacea]
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J Invest Dermatol 117:000-000, 2001

Urtikaria und Quincke-Oedem
T.M. Kündig
Allergolist 2001

Therapie der chronischen Urtikaria und der rezidivierenden Quincke-Oedeme
T.M. Kündig
Therapeutische Umschau, Band 58, Heft 5, 2001, Seite 321-324

Verfahren zur Steigerung der Immunogenität von Impfstoffen
T.M. Kündig
Praxis, Schweizerische Rundschau für Medizin, 89 (37), 1477-84

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Study ZU-GTP-001

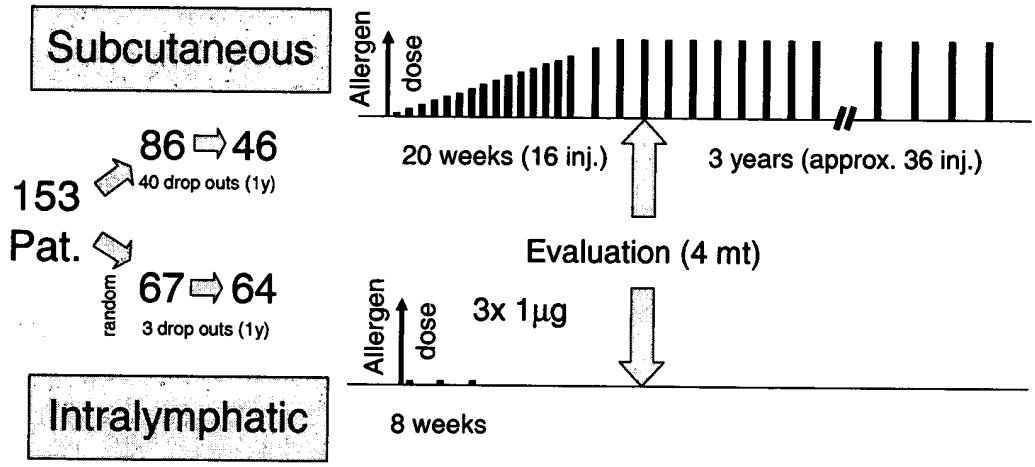
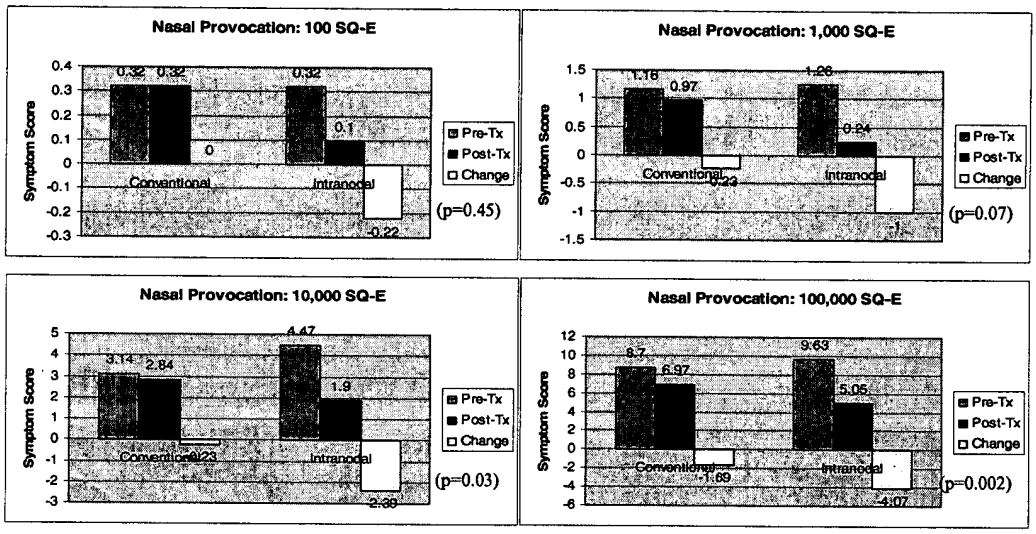


EXHIBIT 3

Nasal Provocation Symptom Scores



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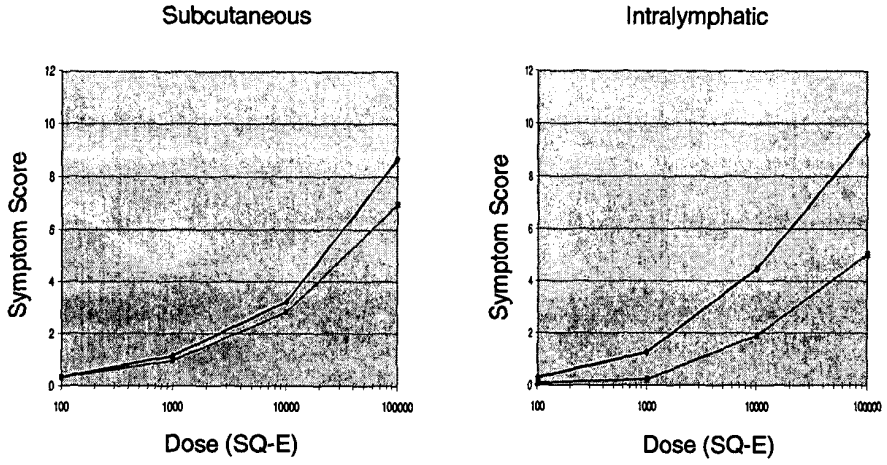
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Intralymphatic
Symptom Score
Dose (SQ-E)
Symptom Score
Dose (SQ-E)

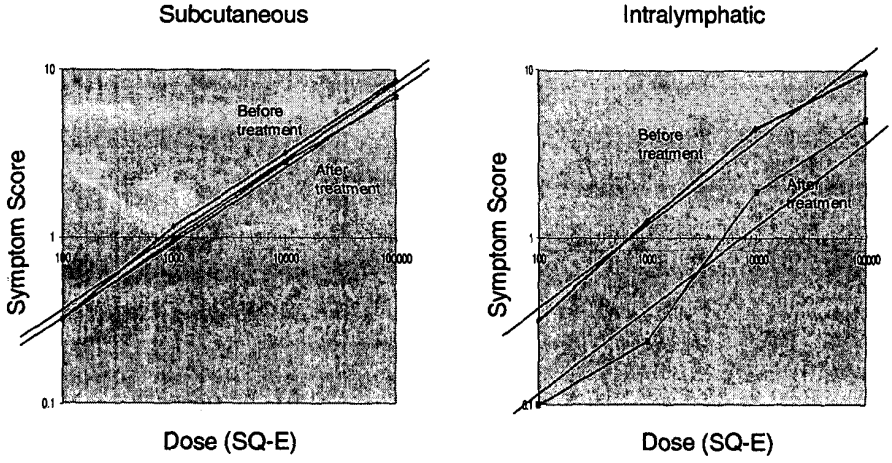
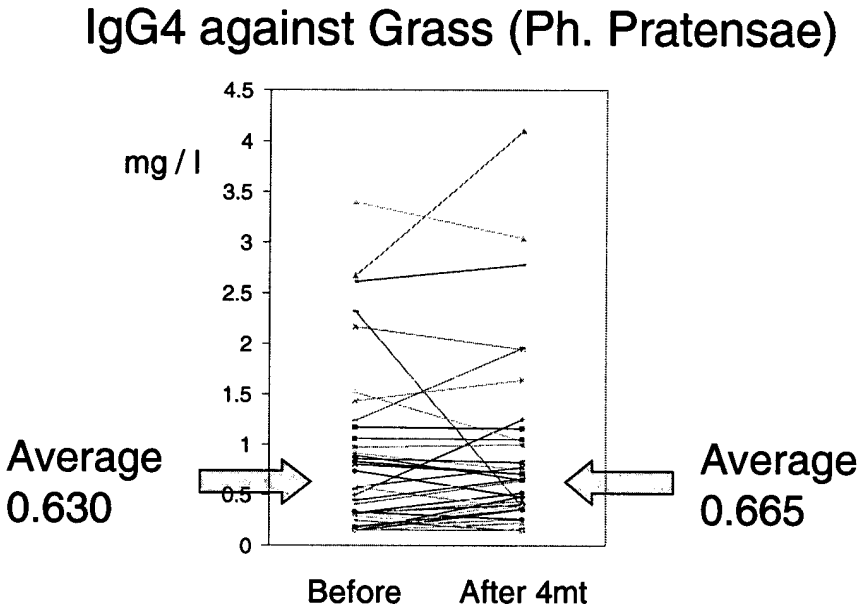

Subcutaneous
Intralymphatic
Before treatment
After treatment
Symptom Score
Dose (SQ-E)
Before treatment
After treatment
Symptom Score
Dose (SQ-E)

EXHIBIT 5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mg / l
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3
2.5
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Average
0.630
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0.665
Before
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EXHIBIT 6

Allergy, 1988, 43, 363–369

Immunotherapy with partially purified and standardized tree pollen extracts

II. Results of skin prick tests and nasal provocation tests from a three-year double-blind study of patients treated with pollen extracts either of birch or combinations of alder, birch and hazel

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Patients allergic to tree pollen entered a 3-year course of immunotherapy (1980–83) with either birch pollen extracts alone (n = 26) or patient-tailored extracts of birch, alder and hazel pollen (n = 27). The clinical and immunological results of this study are published elsewhere. This paper contains an evaluation of skin prick test and nasal provocation test results. There were no significant differences between the two treatment groups concerning these two parameters. In both groups the allergen-specific sensitivity in the skin showed seasonal variations but a significant decrease. During the years of treatment there was also a significant decrease in the specific sensitivity of the nasal mucosa. With the present demands for purification and standardization of allergen extracts it is of practical and economic interest to know that tree pollen-allergic patients showing positive reactions to birch, alder and hazel extracts can be effectively treated using birch pollen extract alone.

Key words: immunotherapy; nasal provocation test; skin prick test; tree pollen allergy.

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Tree pollen allergy is a common cause of springtime rhinoconjunctivitis and asthma in northern Europe (3, 16). In severe cases, treated with immunotherapy, it has been a tradition to use extracts containing a mixture of pollen from birch, alder and hazel (Betula, Alnus and Corylus). These genera are botanically closely related, and most of the patients with springtime symptoms give positive skin reactions when tested with pollen extracts from the three genera separately. Based on aerobiologic studies of pollen frequencies it is known that the exposition to birch pollen is 10 to 100 times higher than to alder and hazel (1). The symptoms of most patients also coincide mainly with the appearance of birch pollen in the air.

The aim of this study is to evaluate whether springtime rhinoconjunctivitis and asthma can be treated as effectively with an extract of birch pollen alone as with a mixture of birch and/or alder and hazel pollen. For this purpose a 3-year double-blind immunotherapy study was performed. The patients were allocated into two groups: one treated with any combination of alder, birch and hazel corresponding to the patient's IgE response (Group I (ABC)) and the other with birch pollen extract alone (Group II (B)). The clinical and immunological results are published separately (8, 9). The results of skin

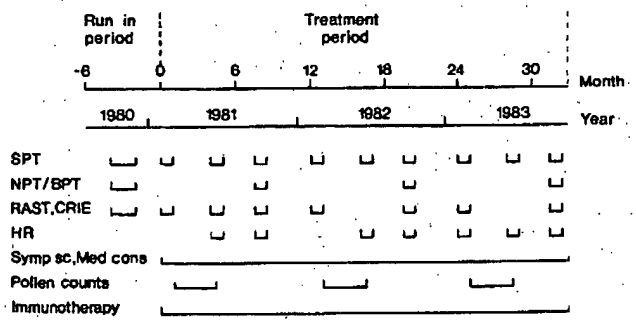

Fig. 1. Study design. SPT: skin prick test; NPT: nasal provocation test; BPT: bronchial provocation test; RAST: radioallergosorbent test; CRIE: crossed radioimmunoelectrophoresis; HR: histamine release; Symp sc: symptom score; Med cons: medicine consumption.

prick tests (SPT) and nasal provocation tests (NPT) are presented here.

MATERIAL AND METHODS

Fifty-one patients with springtime rhinoconjunctivitis and asthma caused by tree pollen entered the study. Based on sex, age, clinical history, SPT, NPT, RAST and CRIE, the patients were allocated into matched pairs. The treatment alternatives were randomly distributed within the pairs into treatment with a tailor-made extract of birch, alder and hazel pollen (n = 27, Group I (ABC)), based on the patient's own sensitivity pattern, and an extract of birch pollen alone (n = 26, Group II (B)). Inclusion and exclusion criteria and patient data are published in detail elsewhere (9). During the years of treatment the patients were followed by repeated *in vitro* and *in vivo* tests as shown in Fig. 1.

Allergen extracts

The allergen extracts used for *in vitro* and *in vivo* tests, as well as for treatment, were partly purified and produced by Allergologisk Laboratorium ALK, Copenhagen, Denmark, and standardized in SQ units. They were lyophilised and used in either aqueous glycerinated or alum adsorbed forms. The same extract was used throughout the study. the specifications of the extracts are given in detail elsewhere (9).

Skin prick test

During the study the patients were tested a total of 10 times, before and after the pollen season and in the winter between the seasons, as can be seen in Fig. 1. The skin prick tests were performed in duplicate and as a titration on both lower forearms using Soluprick SQ® extracts of alder, birch and hazel separately, and with the following concentrations: 0.01, 0.03, 0.1, 0.3, 1.0 and 10 HEP. Glycerine solution served as a negative control and histamine chloride solution in the strength 1 mg/ml as a positive control. Ordinary blood lancets were used for pricking. After an interval of 10 (histamine) to 15 (allergen) min the weals were encircled with a ball-point pen and by transparent tape transferred to mm paper. The results of SPT are given as skin index (SI), calculated as follows:

$$SI = \frac{D(1) \times D(2)}{D(1,his) \times D(2,his)}$$

where D(1) and D(2) are the mutual perpendicular diameters of the weal produced by the allergen preparation measured in millimeters and D(1,his) and D(2,his) are the mutual per-

pendicular diameters of the histamine control, measured in millimeters.

Nasal provocation test

The nasal provocation tests were performed with a mixture of equal parts (in SQ units) of aqueous extracts of alder, birch and hazel. The tests were performed annually and well outside the pollen season, e.g. in October and November, Fig. 1. On each occasion an initial control provocation was performed with saline solution, after which allergen strengths of 0.01, 0.1, 1.0 and 10 HEP were used. The strength of the starting dose was based on the patients's own sensitivity. 0.1 ml of the allergen extract was sprayed into each nostril. Three effect parameters were recorded: nasal patency (as expiratory nasal peak flow), sneezes and secretion. A scoring system was adapted to the outcome of each parameter. One point each was awarded to a 10% decrease in nasal peak flow, 1 sneeze or 0.1 ml of secretion. The scores were added to a total nasal provocation score allowing the NPT results to be given in one figure. A detailed description of the provocation performance and the calculations behind the score are published separately (14).

Statistical methods

The Wilcoxon matched pair rank sum test was used to evaluate differences within the treatment groups and the Mann-Whitney U-test to evaluate differences between the groups (10). All two-sided stochastic probabilities < 0.05 were considered significant.

RESULTS

Skin prick test

Although the patients were skin prick tested with a series of seven allergen strengths ranging from 0.1 to 10 HEP, the statistical calculations are here only presented for 0.3, 1.0 and 10 HEP. The weals elicited by the most diluted extracts were initially small and hence no differences suitable for statistical calculations were achieved. In Figs. 2–4 the results are given graphically and separately for each allergen and treatment group. The pollen frequencies concerning Copenhagen expressed as number of pollen/m³ air/week are also given in the figures.

All the curves expressing weal size have a somewhat uniform appearance, which is most obvious for allergen strengths of 0.3 and 1 HEP. During 1981 there was a continuous decline in weal size, during 1982 an increase, with maximum after the pollen season in the autumn, and during 1983 there was no pronounced change in weal size. Using the Wilcoxon matched pair rank sum test the size of the weals was compared with the initial weal achieved in January 1981. A significant difference ($P < 0.05$) is expressed in the figures with an asterisk above the actual weal size sign. In the majority of comparisons there was a significant decrease of weal size during treatment with the exception of the tests performed after the 1982 pollen season. Differences in weal size from two consecutive tests were calculated using the same statistical method. A significant difference is expressed in the figures with an asterisk on the line between the two test occasions. A visual comparison of the curves for both treatment groups gives a strong impression that they are very much alike for all three allergens. This has also been tested statistically using the Mann-Whitney U-test. Of 90 comparisons of weal sizes from both treatment groups only three showed a slight significant difference ($P < 0.05$). These differences are estimated incidental, and not an expression of a real difference between the two treatment groups.

The pollen curves given in Figs. 2–4 can also be expressed in absolute figures as in Table 1. This gives the total number of relevant tree pollen caught in the spore trap each year. The figures of 54 for 1981, 4259 for 1982 and 1689 in 1983 give a good illustration of the huge natural variation in pollen exposure.

Nasal provocation test

The results of the nasal provocation tests are given graphically in Fig. 5. Only the three

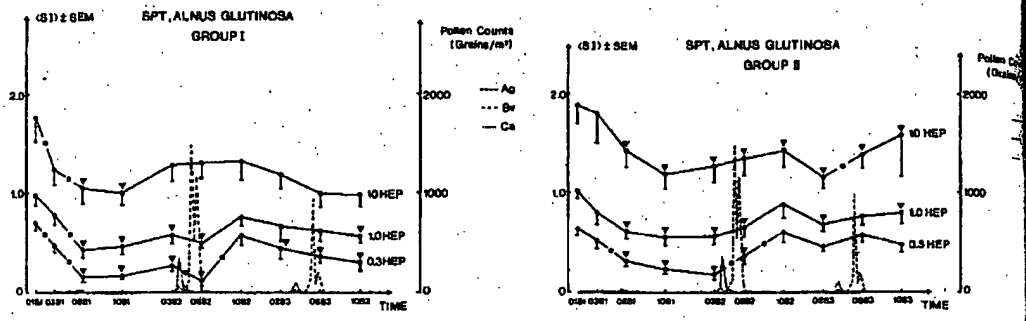

Fig. 2. SPT results for Group I (ABC) and Group II (B) given as SI in both treatment groups using an extract of alder. SI + SEM is given on the y-axis (left) and year and month of the test occasion on the x-axis. Pollen frequencies (number/m³ air/ week) are given on the y-axis (right). Ag: alder, Bv: birch, Ca: hazel. * above an SI value indicates a significant difference ($P < 0.05$) between that value and initial value. * on a line indicates a significant difference ($P < 0.05$) between the two SI values that the line connects.

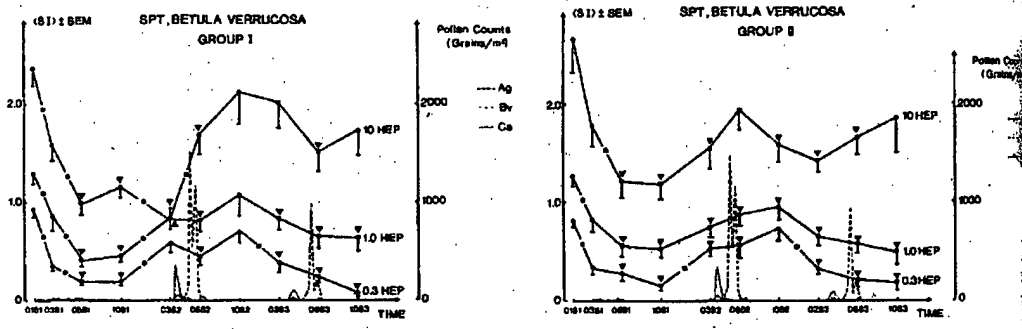

Fig. 3. SPT results given as SI in both treatment groups using extract of birch. Symbols: as Fig. 2.

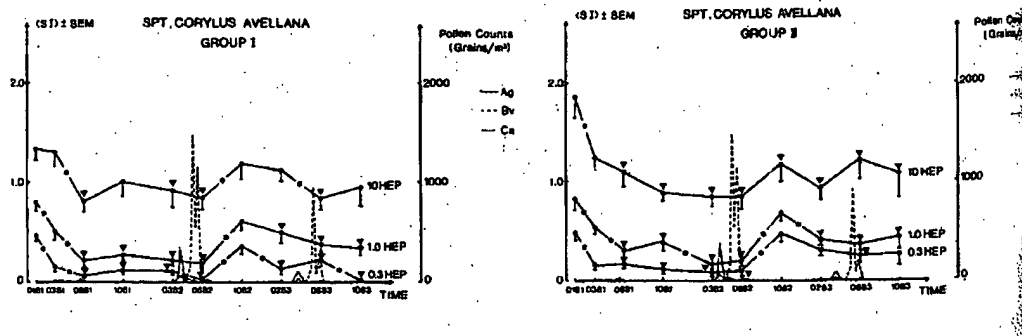

Fig. 4. SPT results given as SI in both treatment groups using extract of hazel. Symbols: as Fig. 2.

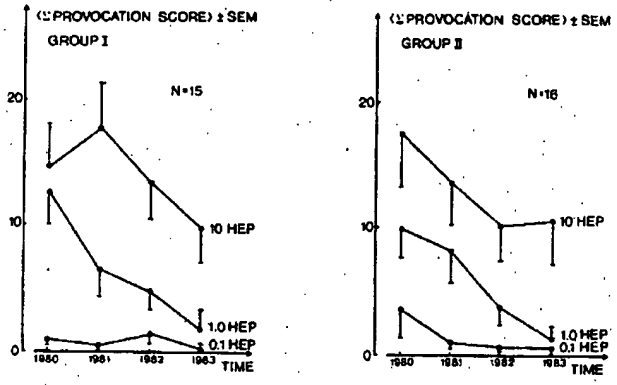

Fig. 5. NPT results in both treatment groups (I (ABC) and II (B)) separately, given as total nasal provocation scores + SEM (y-axis) during the years of treatment (x-axis).

highest allergen concentrations 0.1, 1.0 and 10 HEP are displayed. Complete results from four yearly provocations from 1980–83 were available from 31 patients; 15 in Group I (ABC) and 16 in Group II (B). The results of the provocations are expressed as total nasal provocation scores. As the allergen strength used for provocation was determined by the patient's individual sensitivity the number of patients tested with the different allergen strengths varied during the course of the treatment. Hence, only 13 patients were tested with 10 HEP in 1980 and 16 with 0.1 HEP in 1983 (14). However, almost all patients were tested with 1.0 HEP on each of the four occasions making these tests the most reliable, and the only ones used for statistical evaluations comparing the two groups. A visual comparison of the 1.0 HEP curves for both treatment groups shows a continuous decline in nasal reactivity (Fig. 5). The increase in sensitivity seen in the SPT results from 1982 cannot be traced here.

Comparison of the provocation results using 1.0 HEP for both treatment groups by the Mann-Whitney U-test did not reveal any significant difference, indicating that the two allergen extracts had a similar therapeutic efficacy. Therefore, both groups of 15 and 16 patients were combined when statistical calculations were performed to demonstrate decrease in specific nasal sensitivity during treatment. Using an allergen strength of 1.0 HEP a significant decrease was shown with Wilcoxon matched pair rank sum test in five of six comparisons (Fig. 6).

Table 1

Pollen counts from Copenhagen. Integrated number of pollen/m³/h for the various seasons

	1981	1982	1983
Alder	12	641	188
Birch	36	3495	1477
Hazel	6	123	24
Total	54	4259	1689

DISCUSSION

The efficacy of immunotherapy can be demonstrated by several different methods. Objective methods are of special importance in clinical trials that are not double-blind and without control patients. Change in skin sensitivity is an objective parameter of established value (2, 4, 13, 15) as well as a determination of the sensitivity of the shock organ (1, 5, 12, 15).

In allergic rhinitis change in the sensitivity of the nasal mucous membrane is the most relevant parameter for studying efficacy.

The problems involved in recording symptoms on diary cards are obvious considering the

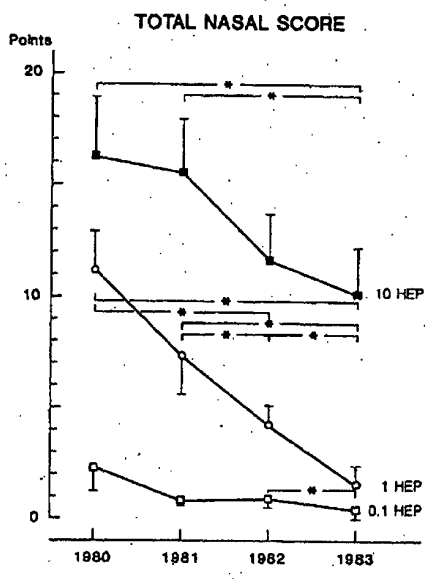

Fig. 6. NPT results of the whole material given as total nasal provocation scores + SEM. Significant differences ($P < 0.05$) calculated with Wilcoxon matched pair rank sum test are indicated with *.

wide variation in natural pollen exposure from year to year. The figures in Table 1 are a good illustration of this problem. However, this can also influence objective parameters, such as skin reactivity which usually increases after the pollen season (6). An effective immunotherapy should eliminate such a seasonal increase in sensitivity. Because of the extremely low pollen counts in 1981 there was probably no natural enhancement of skin sensitivity that year. However, this was clearly detectable in 1982 (Figs. 2–4) in spite of immunotherapy, but not in 1983, perhaps because of immunotherapy.

Only one patient had asthma as the only symptom of allergic disease. In a few other cases asthma was the dominating symptom. Bronchial provocations were performed in these patients, but the total number of tests was too small to allow statistical calculations.

NPT is a rather difficult test as several symptoms need to be recorded. The construction of a total nasal score (14) has probably made the test more sensitive and able to reveal quantitative changes. Using an allergen strength of 1 HEP, which in the majority of patients gave a reaction of acceptable strength, it was possible to demonstrate successively decreasing nasal sensitivity. Clinically, this strength was the most valuable. The number of observations with 0.1 and 10 HEP were much smaller than with 1.0 HEP, and with these strengths it was only possible to show incidental significant differences during treatment (14) (Fig. 6). In the 4 years 15 patients from one group and 16 from the other were provoked. As no difference in therapeutic effect was demonstrated between the groups they were joined to achieve reliable statistical calculations.

The year to year variation in pollen counts did not appear to influence the NPT in the same way as it did the SPT. This indicates that different mechanisms are probably involved in eliciting skin and nasal reactions. With both SPT and NPT a decreasing specific sensitivity during treatment could be demonstrated. However, it was not possible to show any difference between the two treatment groups. This emphasizes the impression formed by studying the pollen frequencies (11): birch is responsible for most of the symptoms of tree pollen allergy in Scandinavia. All the patients in this study were allergic to birch, and hence birch allergen was included in the treatment extracts of both groups. The close botanical relationship between alder, birch and hazel is also the reason for the antigenic identity demonstrated recently (7).

The present demands for purification and standardization of allergen extracts involves much work and expense for the manufacturers. It is therefore of practical importance to show that patients with tree pollen allergy and sensitive to alder, birch and hazel can be effectively treated with an extract of birch pollen alone. This conclusion is based on a clinical trial performed in southern Scandinavia where birch is less prevalent than in the north and it is therefore probably valid for the whole of Scandinavia.

ACKNOWLEDGEMENTS

The authors wish to thank Lone Pedersen, Hanne Steenbgård, Lise Høi Hansen and Annie Lindebod from th
partment of Lung Diseases, C
Gentofte, as well as Ingemo
from the Department of Otol
Hospital, for their careful hai
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IENTS

Lone Pedersen, Hanne S
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Department of Lung Diseases, Copenhagen County Hospital, Gentofte, as well as Ingemo Harryson and Astrid Peterson from the Department of Otolaryngology, Malmö General Hospital, for their careful handling of the patients, and the Aerobiological Group of the Danish Asthma and Allergy Association for the pollen data provided.

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EXHIBIT 7

Allergy 1984, 39, 275–290

Immunotherapy with Alginate-Conjugated and Alum-Precipitated Grass Pollen Extracts in Patients with Allergic Rhinoconjunctivitis

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In order to compare the safety and the efficacy of two grass pollen extracts made from the same starting material, i.e. equal proportions of cocksfoot and timothy, a 5-year study was made on patients with hay fever. One product was a dialysed aqueous extract of pollens which was chemically conjugated to activated sodium alginate (Conjuvac). The other was a pyridine-extracted alum-precipitated crude extract (Allpyral).

Forty patients with classical hay fever were admitted to the study. All gave positive result to prick and nasal/conjunctival testing with extracts of both cocksfoot and timothy grass.

Thirty-five patients completed all 5 years of the study. Seventeen/eighteen Allpyral-treated and 17/17 Conjuvac-treated patients were subjectively improved. There was no significant difference in symptom scores between the two groups. Challenge tests showed a tendency to reduced sensitivity to allergen in the Conjuvac-treated but not in the Allpyral-treated group. The median values for specific IgG and IgE showed the same pattern of reaction as seen in earlier studies of pollen hyposensitization. There was, however, a greater increase of grass-specific IgG in the Conjuvac-treated patients. No serious adverse effects were seen in any of the treatment groups, but there were slightly fewer side effects in the Conjuvac group.

Key words: alginate conjugation; grasspollen; hay-fever; immunotherapy; side effects.

Accepted for publication 9 November 1983

Immunotherapy with specific allergen extracts in the treatment of atopic allergy has now been used for more than 70 years since the introduction of the method by Noon, 1911. There are still many doubts and questions about the efficacy and safety of this treatment, and criticism has been raised against the impurities in crude extracts and the poor standardization of the batches (1, 10, 14). This has encouraged the introduction of a new generation of purified and better standardized allergen extracts by pharmaceutical industries in an attempt to meet the demands from allergists.

Much work has also been done during the last decade with the aim of modifying allergen extracts in ways that will slow absorption or reduce allergenicity with maintenance of immunogenicity. Precipitation of the extract with aluminium hydroxide was one of the earliest techniques for ensuring slowing absorption. This allows a larger dose in each injection than with aqueous extracts and accordingly fewer injections. Because alum-precipitated extracts are as efficacious as aqueous extracts they have been widely used (27). Treatment of allergen with formaldehyde or glutaraldehyde has formed so-called allergoids which in several trials have shown good clinical efficacy and safer administration (22, 23, 26). Another method which has been developed is modifica-

tion of allergens by conjugation to a non-immunogenic polymer such as dextran or polyethylene glycol. This has likewise led to favourable changes in the immunological properties of the allergen, i.e. reduced allergenicity with unchanged or enhanced capacity to suppress specific IgE (19, 21).

The aim of the present study was to compare the safety, immunogenicity and efficacy of such a new, partly purified, alginate-conjugated, freeze dried allergen preparation (Conjuvac) with a traditional, widely used, alum-precipitated, crude extract (Allpyral).

The adjuvant used in Conjuvac, sodium alginate, is a carbohydrate extracted from brown seaweed which when co-valently bound to the allergen has been shown in animal experiments to reduce the allergenicity of grass pollen extract with preservation of the immunogenicity (24, 34). This study was the first in which alginate chemically conjugated to allergen had been used in a clinical trial and therefore intentionally involved only a relatively small number of patients.

Patients with hay fever from two centres for allergology in Sweden participated in the 3-year trial and were studied at regular intervals by means of *in vitro* and *in vivo* tests. Adverse effects of the treatment and symptom scores during the pollen season were registered. Since Conjuvac Two Grass and Allpyral Two Grass are totally different in their appearance a blind design was not practicable.

MATERIAL AND METHODS

Patients

Forty patients (17 females and 23 males), aged 18–43 years (mean 28) with classical hay fever were admitted to the study. Four had seasonal asthma in addition to their rhinoconjunctivitis. Their allergy was proved by a typical case history and positive prick testing with extracts of both cocksfoot (*Dactylis glomerata*) and timothy (*Phleum pratense*). Each patient also gave positive nasal and conjunctival response to challenge with freshly prepared solution of the dialys[ed] aqueous extract of the two grasses. Furthermo[re] they all had a positive radioallergosorbent te[st] (RAST) to timothy pollen allergen. Thirte[en] patients were also clinically birch-sensitive an[d] 15 clinically mugwort-sensitive.

Pregnant women and patients aged less tha[n] 18 years or more than 45 years were exclude[d] as were patients with perennial rhinitis or pe[r]rennial asthma and patients with delayed re[s]sponse to the skin (prick) tests.

The patients were randomly allocated i[n] blocks of two to two treatment groups. Te[n] patients on each treatment were studied in eac[h] of the two centres (Table 1).

All patients gave informed consent befor[e] admission to the trial.

Allergen extracts

All the preparations were produced by Dome[x] Hollister-Stier, Division of Miles Laboratories[,] Stoke Court, Slough, England from grass pol[-] len obtained from Allergon AB, Ängelholm[,] Sweden. The same batches of pollen were use[d] in the preparation of both extracts.

Conjuvac. An equal mixture by weight of polle[n] from cocksfoot and timothy was defatted wit[h] ether and then extracted with modified Coca's solution followed by dialysis by hollow fibre using a nominal 5,000 Dalton cut-off. The term "alpha fraction" was used to describe the resul[t]ant extract. Anaphylactic isoelectric focusing was used to ensure that dialysis had not re[-]moved major proteins.

The procedure used in the conjugation o[f] grass pollen extract to sodium alginate was th[e] mixed anhydride method (24). The produ[ct] was precipitated, washed free of unconjugate[d] protein, redissolved and lyophilised. The Con[-]juvac so produced was approximately fou[r] parts by weight sodium alginate to one par[t] allergenic protein (34).

Allpyral. The same mixture of equal part[s] cocksfoot and timothy pollen was extracted i[n] pyridine and precipitated with aluminium hy[-]droxide. The precipitate was filtered an[d]

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PNU determination. The Dome standard procedure was used to determine the protein nitrogen units (PNU) contained in the pollen extract. The proteins were precipitated with phosphotungstic acid and the nitrogen in the precipitate was assayed by the micro-Kjeldahl method (18). The units of protein are related as follows: 100 PNU = 1 μg protein nitrogen which represents on average 6.25 μg protein.

Allergenic activity. The potencies of the stock solutions of the extracts were also measured by RAST inhibition.

Treatment

Injections started at the beginning of January 1978 and continued to the end of May 1978. They were restarted in November 1978 and continued to the end of May 1980. Allpyral treatment sets consisted of multiple dose vials of 100, 1,000 and 10,000 PNU/ml strengths. Conjuvac treatment sets consisted of single dose vials of lyophilised Conjuvac of 10, 100, 1,000 and 10,000 PNU/ml strengths when reconstituted.

Dosage. Since this was the first clinical study of Conjuvac and its first administration to man, the dosage schedule started cautiously at 1 PNU, approximately doubling the dose on subsequent weekly injections to a maintenance dose of 10,000 PNU given once monthly. In the second year Conjuvac injections started at 5 PNU, since experience indicated that this would be tolerated by the patients. A lower maintenance dose (5,000 PNU), however, was adopted as serum grass-specific IgG levels during the first year suggested this would be adequate for equivalence with those obtained by the Allpyral-treated patients. The maintenance dose was halved during coseasonal treatment in 1979 and was then raised to 10,000 PNU after the season. The Allpyral dosage schedule was that currently employed in Sweden, i.e. starting with 10 PNU, approximately doubling the dose on subsequent weekly injections to a maintenance dose of 10,000 PNU or else the highest tolerated, given once monthly with a similar coseasonal dosage reduction as indicated above.

Before an injection was given the patient was asked whether any reaction (local or systemic) occurred after the previous injection. After each injection the patients remained in the clinic for 30 min and the diameter of local reactions was measured. Modifications to the dosage schedule were made if a local reaction at the injection site was greater than 5 cm in diameter or if any systemic reaction occurred.

Patients received no concurrent hyposensitization with any other allergen extracts.

Symptomatic treatment during grass pollen season was prescribed if needed and consisted mainly of antihistamine tablets (Polaramine® 2 mg containing dexchlorpheniramini maleas) and eye drops (Antasten-Privin® containing antazoline sulphate and naphazoline nitrate). If this treatment was insufficient beclomethasone dipropionate nasal spray (Becotide nasal®) was added with a maximum of 4 doses per day.

Skin prick test

For skin testing, alpha fractions of timothy and cocksfoot were dissolved in glycero Coca's solution at a strength of 1% by weight of pollen before dialysis. Fresh material from the same stock batches of the lyophilised extracts was used throughout the study. Birch testing was made with the standard Dome diagnostic solution of *Betula pendula*. In addition Conjuvac Two Grass 100, 1,000 and 10,000 PNU/ml were skin-tested in the patients to ensure before their use in treatment that they were free from excessive skin reactivity. As positive and negative controls 1% histamine acid phosphate and Coca's solution were used.

Patients were skin tested on admission to the trial and 2 weeks after the end of the grass pollen season in the first year. In the second year they were tested 1 week before and 2 weeks after the grass pollen season and in the third year of the trial 1 week after the last injection before the pollen season.

The skin prick was made on the volar side of

the forearm with an ordinary blood lancet placed at an angle of approximately 60° to the skin. With the lancet tip inside the allergen drop just entering the epidermis it was slightly raised and then withdrawn. After 60 sec the drops were wiped off and after 15 min each wheal and flare was carefully outlined with a ballpoint pen and using sellotape the outlines were transferred to squared millimetre paper.

For each extract in each patient the ratio wheal area of allergen solution/wheal area of histamine solution was calculated. The lowest wheal area for the histamine reference accepted was 3 mm.

The tests were performed single but by the same nurse at each centre. The coefficient of variation was within 10%.

Provocation tests

The allergen used throughout the study was a single batch of lyophilised mixture of the alpha fractions of cocksfoot and timothy in equal proportions by weight of pollen before extraction and provided in vials each containing 24,000 PNU. Solutions of this material were prepared in physiological saline at 5, 50, 500, 1,000 and 5,000 PNU/ml and a measured amount used on each occasion. Physiological saline was used as a negative control before allergen provocations.

Conjunctival test. One drop of the weakest allergen solution was instilled into the conjunctival sac of one eye. If no redness of the conjunctiva was observed in 10 min and the patient did not feel any itching in the eye the provocation proceeded with a stronger concentration and so on until a positive result was obtained.

Nasal test. Each instillation of allergen solution in the eye was directly followed by nasal test. With the patient's head held backwards 0.1 ml of the weakest allergen solution was placed in one nostril and the reaction was registered after 10 min. If sneezing, nasal discharge and/or blockage occurred the test was considered as positive. If the reaction was negative the provocation continued with the next stronger allergen concentration and so on until a positive result was obtained.

Patients underwent conjunctival and nasal testing on admission to the study, 1 week after the last injection before the 1978 grass pollen season and approximately 2 months after the end of the 1978, 1979 and 1980 grass pollen seasons. The test nurse did not know the results of former conjunctival and nasal tests.

Serological studies

Serum samples were analysed in one run at the end of each year of the study in the Research Laboratories at Stoke Court. At the end of the second and third years the previous years' samples were re-assayed to ensure reproducibility of results – these were within 10% of the previous result. One operator assisted in the work throughout the 3 years. The same batch of grass pollen extract was used for RAST discs and specific IgG and IgA assays throughout the study. Serum was collected from each patient on admission to the trial, after every fourth injection during the first preseasonal treatment and a couple of weeks before and after the grass pollen season 1978, 1979 and 1980. Samples were subsequently assayed for total IgE (15), grass specific IgE (16) and grass specific IgG (11). The first-year samples were also assayed for total IgG (9), total IgA (9), grass specific IgA (7) and alginate specific haemagglutinating antibody.

Cellular studies

Lymphocyte transformation studies were performed by Dr Per Broman on blood samples collected from the 20 trial patients in Huddinge. The results of these studies will be published elsewhere.

Diary cards

Each patient kept a daily record of symptoms and medication required during the period May to August each year. Symptoms scored were nose: blocked, running, sneezing, itching; eyes: itching, running; lungs: wheezing and skin: itching. Each symptom was graded as follows: 0 = no symptoms, 1 = mild symptoms,

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= moderate symptoms and 3 = severe symptoms. The daily maximum recorded score for each target organ for each patient was obtained. For example, if nasal blockage was scored 2, running 1, sneezing 3 and itching 0 by a patient, the maximum nasal score for that day was 3. The sum of such scores for the four target organs was defined as the "symptom score" for that patient on that day.

Medication was recorded as the number of antihistamine tablets, the number of times eye drops were used and the number of times other symptomatic medication was taken. The sum was defined as the "medication score" for that patient on that day.

Pollen counts

Pollen counts for the two centres were obtained during the three relevant grass pollen seasons and expressed as mean number of grass pollen grains/m^3 air/day.

Pollen grains were collected in Burkard Recording Volumetric Spore Traps placed in the centre of Gothenburg and on the roof of Huddinge Hospital respectively. Both traps were placed about 25 m above the ground. Pollen counting was performed at the Palynological Institutions at the Gothenburg and Stockholm Universities. All the patients were living within a radius of 30 km of the spore traps.

Statistical methods

All data was stored on an IBM 4331 computer and analysed using Fortran programmes. Most of the data generated by the trial did not follow a normal distribution and thus were analysed using non-parametric methods such as signed rank tests and the Mann-Whitney U test (4).

A non-standard method was used on the serological data to calculate confidence intervals for the median (30).

Diary card data were transformed towards normality using the Inverse Normal Scores transformation (4).

All *P*-values presented refer to 2-tailed tests.

RESULTS

The following patient parameters on admission to the trial were compared between the two treatment groups and the two centres by analysis of variance or chi-squared as appropriate: Total IgA, IgG and IgE, grass specific IgA, IgG and IgE, birch specific IgE, sex, age, duration of disease, seasonal asthma and history of atopic eczema. Results between centres were sufficiently similar to justify pooling of results and between treatment groups sufficiently similar to justify comparison of treatments. Of the 40 patients who took part, 35 completed the full 3-year study, 17 Conjuvac and 18 Allpyral. Four patients dropped out after moving to other areas: two from the Conjuvac and one from the Allpyral group after first year of study and one more from the Conjuvac group after second year of study. One patient from the Allpyral group interrupted the treatment at an early stage during the first year, feeling as he said: "more discomfort with the injections than with the pollen season as such". No adverse reactions, however, were observed in this case.

Dosage

A summary of the median total doses administered during each of the 3 years is presented

Table 1

Parameters on admission of patients by treatment group

Parameter	Conjuvac	Allpyral
n	20	20
Age, median years and (range)	27.5 (18–37)	26.0 (19–43)
Males	11	12
Females	9	8
Rhinitis only	16	20
Rhinitis and asthma	4	0
Duration of disease, median years and (range)	10 (2–20)	11 (5–25)
Clinically birch-sensitive	6	7
Clinically mugwort-sensitive	8	7
Clinically mould-sensitive	0	0
History of atopic eczema	3	1
Previous immunotherapy (grass) > 3 years ago	2	3

Table 2

Median total doses given to patients who completed 3 years study. Inhibitory activity by RAST related to first British Cocksfoot (*Dactylis Glomerata*) reference extract in brackets

	n	PNU (RAST)		
		1st year	2nd year	3rd year
Conjuvac two-grass	17	25554 (14587)	39025 (18895)	57500 (27840)
Allpyral two-grass	18	40090 (12949)	82720 (16238)	65000 (1762)

in Table 2. September 1979 has been taken as the end of the second year of the study. The doses are expressed in terms of total PNU and total activity by RAST.

During the first 2 years for which fresh batches of Conjuvac and Allpyral were prepared each year, the doses of Conjuvac and Allpyral administered were comparable in RAST terms. During the third year, in which the same batches of Conjuvac and Allpyral as were provided for the second year were used, these extracts were 12–18 months old and the RAST activity of the Allpyral had declined during this time. The dosage schedules were originally defined in PNU terms and the patients were treated with similar doses of either Allpyral or Conjuvac. However, when dosage is redefined in RAST terms, the Allpyral patients received only about one sixteenth of the dose received by Conjuvac patients in the third year.

Skin tests

Since Conjuvac had not previously been administered to patients all 40 patients were prick-tested on admission to the trial with Conjuvac Two Grass of three strengths – 100, 1,000 and 10,000 PNU/ml in addition to alpha fractions of timothy and cocksfoot. The means of wheal area ratios showed that the alginate conjugation had resulted in an approximately 3-fold reduction in the allergenicity of the pollen extract as measured by skin test reactivity. The two treatment groups behaved similarly in this respect (Table 3).

Both treatment groups showed statistically significant decreases in "wheal area ratio" between admission and 2 weeks after the first grass pollen season and also between this last point of time and 1 week before the second grass pollen season (Fig. 1). These decreases occurred for both timothy and cocksfoot extracts. Allpyral patients also showed a significant increase in histamine wheal area between the first and second years. Between the post-treatment skin tests in the second and third years further decreases in "wheal area ratio" were seen for both grasses and treatment groups. These changes were much smaller than those seen earlier in the trial. The individual changes in "wheal area ratio" from one skin testing occasion to the next which were statistically significant are marked with asterisks. Both treatment groups appeared to show similar changes in "wheal area ratio" throughout the trial.

Provocation tests

Unfortunately, provocation tests were only performed consistently at one centre. Therefore only results for the patients in Huddinge can be given. Fig. 2 shows the individual results of

Table 3

Means of wheal area ratios at skin testing in the two treatment groups on admission to the study

Extract		Conjuvac	Allpyral
Timothy	3,000 PNU	3,47	4,79
Cocksfoot	2,500 PNU	4,29	4,99
Conjuvac	100 PNU	0,22	0,33
Conjuvac	1,000 PNU	1,0	1,39
Conjuvac	10,000 PNU	3,3	3,76

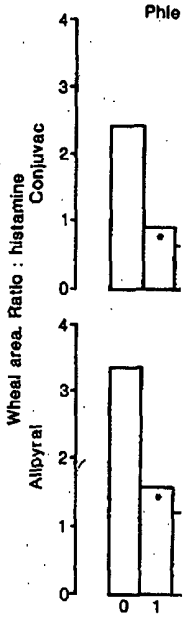

Fig. 1. Wheal area ratios for

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2 = After treatment
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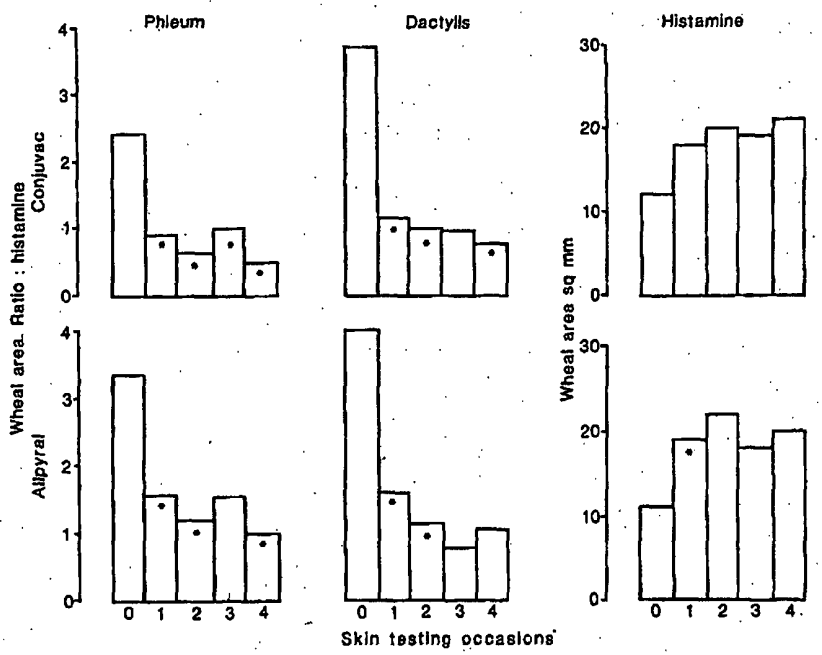


Fig. 1. Wheal area ratios for skin testing with timothy and cocksfoot extracts. Actual wheal area for histamine skin testing.

Time 0 = On admission to trial
1 = 2 weeks after grass pollen season 1978
2 = After treatment 1979
3 = 2 weeks after grass pollen season 1979
4 = After treatment 1980.

* indicates statistically significant change ($P < 0.05$) from previous test.

nasal provocation test in the two treatment groups. The lowest concentrations of allergen required to give a positive response are plotted. There is a tendency to reduced sensitivity to allergen in the Conjuvac-treated group which is hardly demonstrable in the Allpyral group. No statistical analysis has been made considering the limited number of patients studied. The results for conjunctival tests were similar.

Serological tests

There were no significant changes in total or grass specific IgA, total IgE, total IgG or alginate specific IgM during first year of treatment. Subsequently, of these parameters, only total IgE was followed. Conjuvac patients had a greater fall in total IgE ($P < 0.05$) over the second year compared to Allpyral patients, but the median value for total IgE remained fairly constant in both treatment groups during the third year (Fig. 3).

Grass specific IgE showed an initial, though not statistically significant, rise during the preseasonal treatment in 1978 in the two groups and there was an inhibition of the seasonal increase that usually follows exposure to environmental pollen in non-treated patients (Fig. 3). The grass specific IgE levels fell in both groups below the initial values after the second preseasonal treatment and there was still no postseasonal rise.

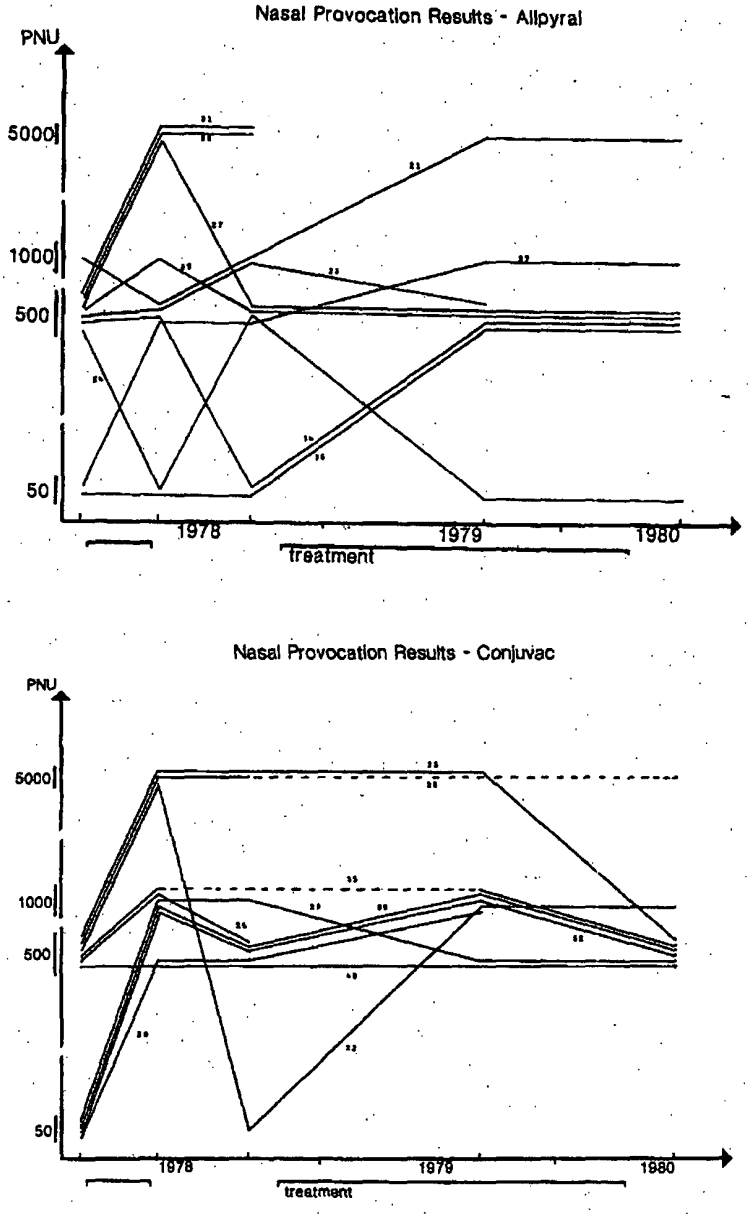
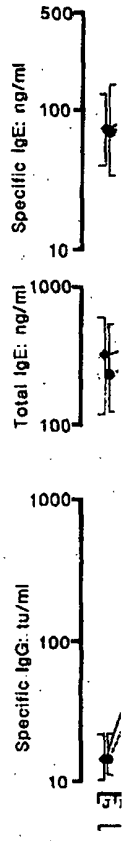


Fig. 2. Nasal provocation test results in Allpyral- and Conjuvac-treated patients (Huddinge patients only). Concentration required to give a positive response.



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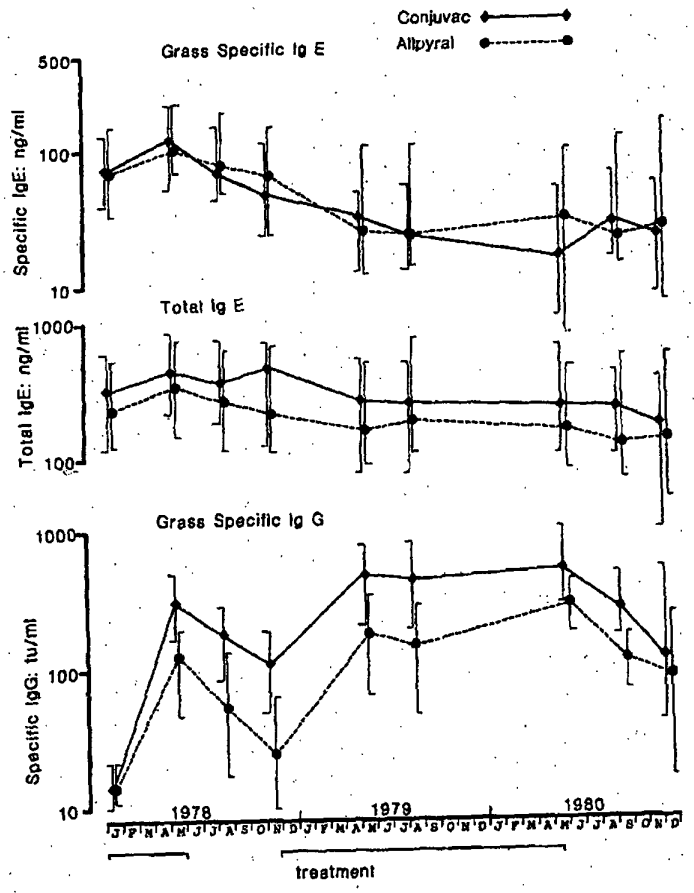
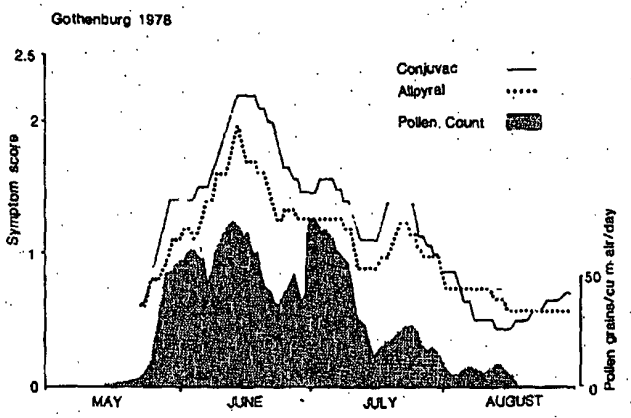


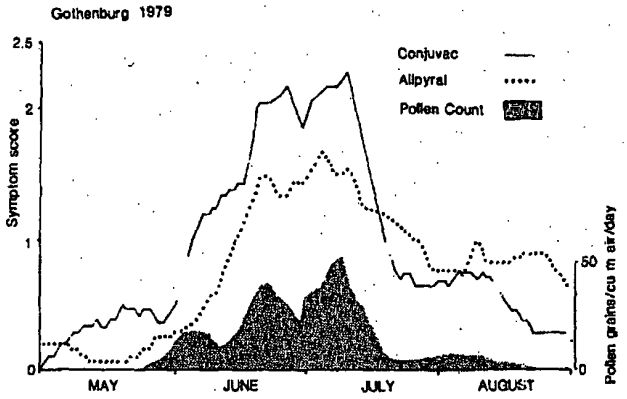
Fig. 3. Median values for grass specific IgE, total IgE and grass specific IgG. Confidence intervals widen as patient numbers decrease from 38 to 32 over the 3 years of the study.

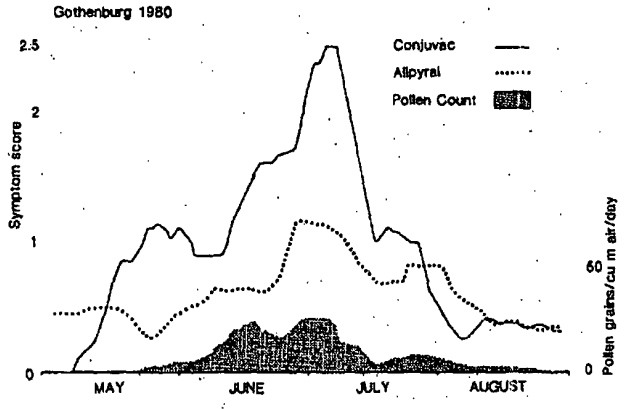
Grass specific IgG markedly increased during the preseasonal treatment 1978 in both groups; however, the increase in the Conjuvac-treated patients was significantly greater than that seen in the Allpyral-treated group ($P <$ .005). The difference between the groups is not significant later in the trial.

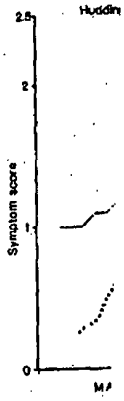
In Fig. 3 the median values for grass specific IgG can be followed over the 3 years of study. After the initial rise during the preseasonal treatment the levels fell off when no treatment was given from June to November 1978, rose again during the second preseasonal injection period, fell off less dramatically during the second pollen season when dosing continued at 50% maintenance level, remained fairly constant during maintenance therapy and then declined rapidly after end of immunotherapy in May 1980.

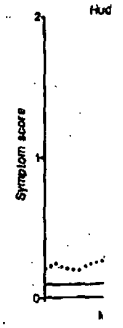
When looking more closely at the 3 years' data, in order to see if there were any differences between the treatment groups, one factor which did arouse interest was an apparent difference in the rate of grass specific IgG re-

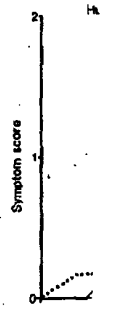












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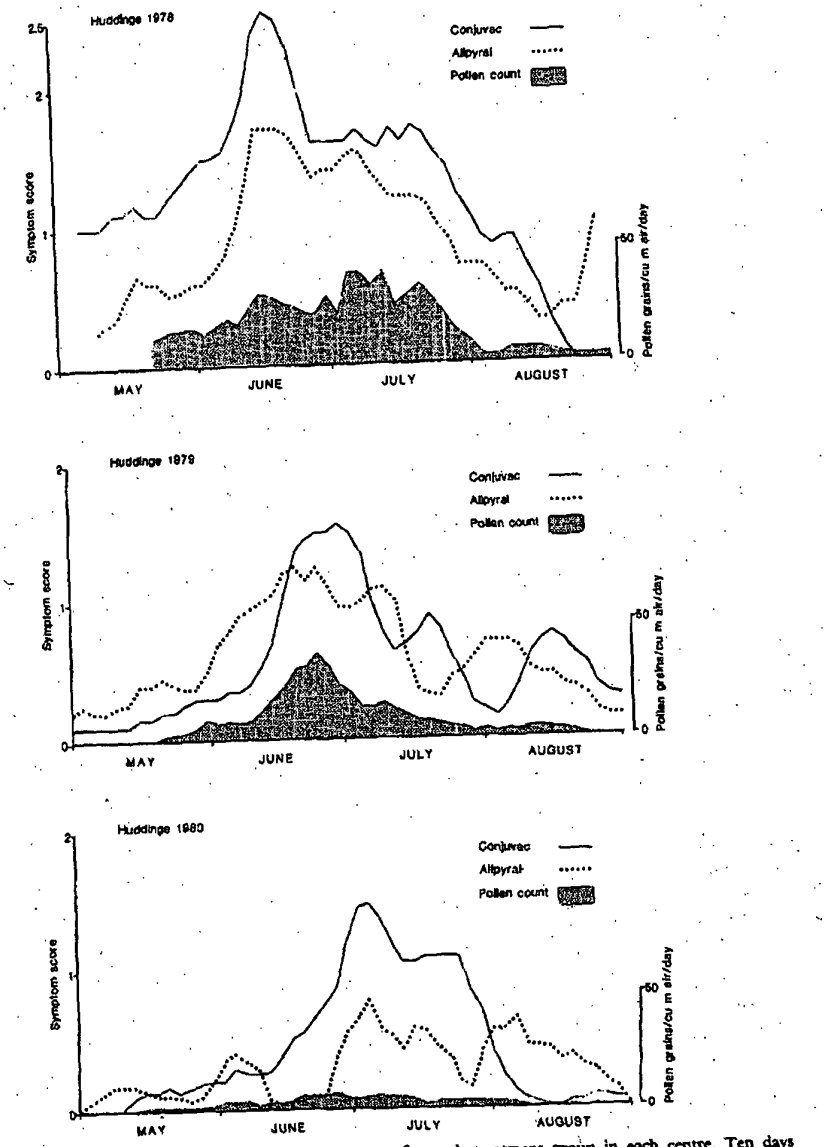

Fig. 4a,b. Ten day running mean of mean symptom scores for each treatment group in each centre. Ten days running mean of daily grass pollen count also shown.

ponse to therapy. In an attempt to clarify this we have examined data from only those patients who completed all 3 years of the study. Of 17 Conjuvac-treated patients 12 achieved a 10-fold increase in grass specific IgG during the first preseasonal course of injections and a further three during the second and third years. In the Allpyral group only 4/18 achieved that level

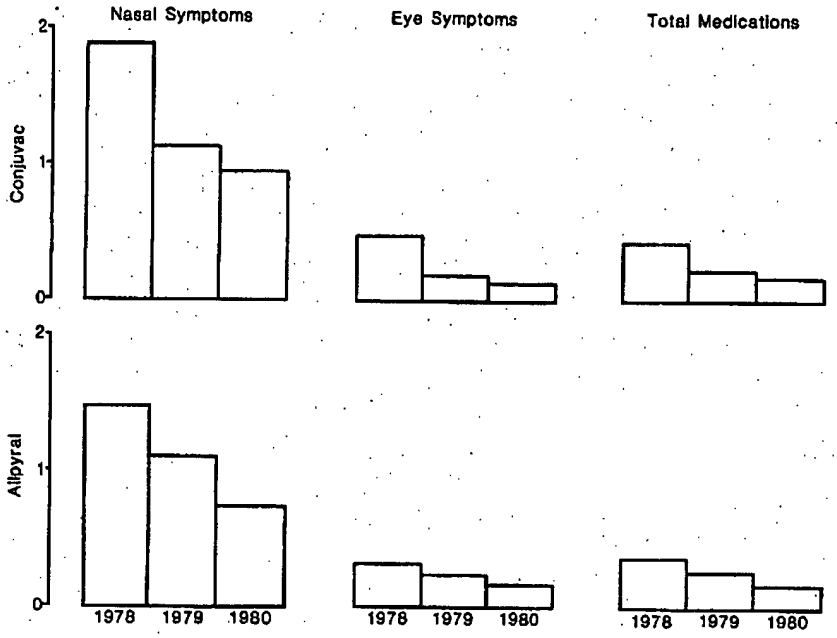

Fig. 5. Mean symptom and medication scores for Conjuvac- and Allpyral-treated groups. Treatment group differences not statistically significant.

during the same time and a further nine during the second and third year.

Analysis of diary cards

Fig. 4a,b show plots of 10-day running means of mean symptom scores for both treatments, and 10-day running means of grass pollen counts in both centres for 1978, 1979 and 1980. Some patients left their domestic area during the grass pollen season and the data on the diary cards were omitted from the analysis during these days.

Mean symptom and medication scores for Conjuvac- and Allpyral-treated groups are illustrated in Fig. 5. Treatment group differences were not statistically significant. Scores decreased year by year but grass pollen counts were lower in the third year than in the first two.

Subjective assessments

At the end of each pollen season both the patient and clinician were asked to assess the patient's response to therapy on a 5-point rating scale. In each of the 3 years there were no statistically significant differences between treatment groups. Of the patients who completed the 3 years, 17 of 18 on Allpyral and all 17 on Conjuvac registered some degree of improvement during the study.

Adverse effect

Table 4 shows percentage of patients needing no dosage schedule modifications because of adverse effects.

A number of patients experienced systemic adverse reactions during the study, in some cases in more than one target organ during a

Table 4

Percentage of patients needin modificati

	Conjuvac n	
Year 1	20	45%
Year 2	18	78%
Year 3	17	71%

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Number of patient

Target organs	
Eyes	
Nose	
Lungs	
Skin	
Throat	
Tiredness	
Other	
No systemic side effects	
Total number of patients represented	

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Table 4

Percentage of patients needing no dosage-schedule modifications

	Conjuvac		Allpyral	
	n		n	
Year 1	20	45%	20	20%
Year 2	18	78%	18	56%
Year 3	17	71%	18	61%

single episode. Table 5 shows the number of patients in each treatment group experiencing adverse effects in each target organ over the 3 years of treatment. During the first year one patient treated with Conjuvac had slight asthma within half an hour after an injection and was given adrenalin. During the third year of treatment another Conjuvac-treated patient had slight oedema of the throat, generalized erythema and tachycardia within 10 min of the injection and was given adrenalin. Otherwise the reactions were mild and delayed and came after 6 h or more.

DISCUSSION

Previous studies by other investigators have indicated that immunotherapy with purified timothy pollen extracts may give a better clinical protection than that with crude extracts (3, 13, 20). In this study, where one of the extracts was partially purified, such a comparison cannot be made as the two extracts used are coupled to different adjuvants, and may influence the results. Many researchers have demonstrated fewer systemic side effects with extracts coupled to alum (25, 31). Yet anaphylactic shocks in connection with immunotherapy with alum-precipitated extracts have been reported (32, 35, 37). Therefore there is a need for better adjuvants which reduce the allergenicity of the allergen extract while preserving immunogenicity, thereby making the immunotherapy safer.

The total median doses of the two extracts administered during the first 2 years were comparable in RAST terms. In the third year, however, the Allpyral extract, which was not lyophilised, apparently had undergone a change as the RAST activity had declined considerably. Inhibitor activity by RAST should be a reasonably relevant parameter to determine the potency of an allergen extract (10, 14, 38). Yet, judging from immunoglobulin levels, the immunogenicity of Allpyral seems to be intact in spite of the low RAST value. This is difficult to interpret unless the loss of RAST activity in the

Table 5

Number of patients with systemic side effects in different target organs for each year of the study

Target organs	Conjuvac			Allpyral		
	year 1	year 2	year 3	year 1	year 2	year 3
Eyes	1			1	4	1
Nose	2	1		6	5	5
Lungs	1			2	1	
Skin	1		1	1		1
Throat			1	1		
Tiredness				1		
Other						1
No systemic side effects	10	17	16	7	11	15
Total number of patients represented	15*	18	17	15*	18	18

* Remaining data lost in an office fire.

vials retained for stability tests by the supplier was for some reason more rapid than that of the vials from the same batch used for treatment and stored in Sweden. Nevertheless, this finding illustrates the importance of checking the stability of the allergenic activity. In the present study, as well as in previous from other investigators, freeze-drying of the allergen preparation has been shown to increase the storage stability (2, 5, 13).

The comparison of the allergenicity, measured by skin test, of the alpha fractions of timothy and cocksfoot extract and the Conjuvac two-grass showed that the alginate conjugation had resulted in an approximately 3-fold reduction in allergenicity at the same PNU-concentration. In spite of that the immunogenic capacity measured by influence on specific immunoglobulin levels was preserved and the pattern was similar to that of Allpyral. The increase in grass specific IgG was even significantly greater and more rapid in the Conjuvac-treated group than in the Allpyral-treated group. Consequently, Conjuvac had a superior immunogenicity regarding IgG antibodies compared to Allpyral in this study.

Taylor et al. (34) have also shown in mice studies that the conjugation of alginate to grass pollen extract led to markedly enhanced specific IgG response while there was little effect on the IgE antibody levels. Hamaoka et al. (17) have, also in mice studies, demonstrated that alum-adsorbed antigen favoured IgE rather than IgG responses, whereas the reverse was true for antigens included in Freund's complete adjuvant. This observation has, however, not been confirmed in man.

Regarding the clinical efficacy of immunotherapy with Conjuvac compared to Allpyral, no definite conclusions can be drawn.

The mean symptom and medication scores for Conjuvac- and Allpyral-treated groups showed decreases over 3 years in both groups, but the differences between the groups were not statistically significant. In all 3 years the pollen count was low with relatively few days when the count exceeded 50 grains/m³ air/day, but the decrease of scores during the third pollen season could partly be due to the extremely low grass pollen counts that summer.

As the provocation tests only include half of the patients, no statistical calculations have been performed. However, a general tendency to increased tolerance for grass pollen by both conjunctival and nasal testing was only evident in the Conjuvac-treated group.

In several previous studies nasal and/or conjunctival provocation tests have been used to assess the efficacy of immunotherapy with grass pollen in hay fever (3, 8, 12, 13, 28). There was a general tendency towards increased tolerance after therapy, but often with a fairly low significance. It must be emphasized, however, that challenge tests are subject to many variables and "even with the variables controlled as far as feasible, a challenge test is at its best only semi-quantitative" (2).

The skin sensitivity to both timothy and cocksfoot extracts was significantly and similarly reduced in both the treatment groups already after the first preseasonal treatment. The significant increase in histamine wheal area between the first and second years in the Allpyral-treated patients and the same tendency in the Conjuvac-treated group is difficult to explain. One simple explanation could be that the histamine solution had a lower activity the first year. If so, the greatest part of the reduction in allergen sensitivity between the years disappears. If, on the other hand, the histamine solution has been equal in strength on the different test occasions the unspecific skin reactivity may have increased as a result of the immunotherapy or/and the natural pollen exposure. A hint in that direction could be that one patient from each treatment group demonstrated cold urticaria during the winter seasons when perennial immunotherapy was given. After the end of the treatment their tendency to cold urticaria disappeared. A high frequency of urticaria has been shown in another recent study (29).

Several other investigators have found a reduction in skin sensitivity to grass pollen after immunotherapy with aqueous- or alum-precipitated extracts, but no information about the reproducibility of the histamine wheal area has been given in these studies (3, 28, 33, 36).

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In the Conjuvac-treated group 33% experienced mild systemic side effects (SSE) during the first year's preseasonal treatment but only 6% during the second year. In the Allpyral-treated group the corresponding numbers were 38% vs 39%. It must be pointed out, however, that the maintenance dose in the Conjuvac group did not exceed 5,000 PNU during the second year, while the corresponding dose in the Allpyral group was 10,000 PNU. During the third year, when the maintenance dose was 10,000 PNU in both groups, SSE occurred in 6% of the Conjuvac patients and in 17% of the Allpyral patients.

In a study by Ransom (31), concerning the frequency of reactions to alum-precipitated ragweed extract, SSE occurred in 12.5% of the patients during the first year but only 4.5% during the second year. Similarly, Østerballe (29) reported SSE in 33% of the patients receiving alum-precipitated grass pollen extract before the first season, but only in 3% during subsequent perennial therapy. Systemic side effects thus occur above all during the increase in dosage levels and are highly dosage related (6). Frostad et al. (13) who used lower doses than in the studies above did not report any SSE. The new generation of purified and standardized extracts is often very potent and therefore demands a more cautious dosage. Alginate seems in this study to be an adjuvant which gives a somewhat better protection than aluminium hydroxide against SSE in connection with immunotherapy with grass pollen.

Results from this preliminary study have been sufficiently encouraging to warrant larger scale clinical trials.

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